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13. SUPPLEMENTARY NOTES

14. ABSTRACT- Breast cancer is considered to be initiated by mutations in a limited population of undifferentiated cells termed stem cells that "sit' at the top of the mammary epithelial hierarchy. Over-expansion of the stem cell population leads to increased numbers of mutated stem cells that initiate and maintain tumors that eventually metastasize. Novel strategies to decrease the proliferation and promote the elimination of tumor-initiating stem cells are warranted. Our current studies test the hypothesis that dietary factors confer protection from breast cancer by preventing the expansion of stem/progenitor cells with tumorigenic potential. We established female mice transgenic for the oncogene Wnt-1, which develop tumors, as a model system for dietary prevention of mammary carcinogenesis. Mice were fed AIN-93G based isocaloric diets that differed only by protein source, namely casein (CAS) and soy protein isolate (SPI). SPI was used as a paradigm for healthy foods, given the linkage of decreased breast cancer incidence with high consumption of soy-rich foods. We found that dietary exposure to SPI resulted in lower tumor incidence in Wnt-Tg female mice. Studies are in progress to isolate and characterize stem cells from tumors of Tg mice fed the two diets to evaluate the basis for the tumor protection.

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INTRODUCTION

Breast cancer is considered to be initiated by mutations in a limited population of undifferentiated cells, termed stem cells that 'sit' at the top of the mammary epithelial hierarchy. The normal stem cell population is tightly regulated and its overexpansion can alter the balance of self-renewal and differentiation, leading to aberrant proliferation and increased risk of mutations. Since mutated stem cells are key to the etiology of breast cancer and are capable of maintaining tumors that can eventually metastasize, novel strategies to decrease the proliferation and promote the elimination of cancer stem cells are warranted. Our current studies test the hypothesis that dietary factors confer protection from breast cancer by preventing the expansion of stem/progenitor cells with tumorigenic potential. The diet/stem cell cross-talk underlying the ontogeny of mammary tumors has not been previously addressed in the mammary cancer field. In these studies we use soy protein as a 'paradigm' for healthy foods, since epidemiological studies have linked decreased breast cancer incidence in Asian women with their high consumption of soy-rich foods. The findings from our studies are anticipated to provide new paradigms, identify molecular targets, and offer easily adoptable lifestyle changes for the early prevention, better treatment, and improved prognosis of breast cancer.

BODY

The major objective of the current studies is to establish the role of diet in the regulation of cancer stem cells leading to the primary prevention of breast cancer. The linkage of diet and stem cells in mammary tumor development initiated by aberrant Wnt signaling, the latter a major contributor to stem cell expansion (1, 2), was addressed by using mammary tumor virus (MMTV)-Wnt-1-transgenic mice (Tg). The study has two Specific Aims. Aim 1 seeks to establish the mammary tumor-prone Tg female mice (3) as a model to evaluate the protective effects of soy-based diets against Wnt-induced mammary tumors. The prediction is that soy protein isolate (SPI), relative to control diet Casein (CAS), will significantly decrease the incidence of mammary tumors and the occurrence of malignant tumors in adult females. Aim 2 will examine if accumulation of the mammary stem/progenitor cell population associated with increased Wnt signaling in heterozygous Tg females is decreased with dietary intake of SPI, relative to CAS. The prediction is that the tumor stem cell population will be smaller in the SPI-fed relative to the CAS-fed mice. To date, Aim 1 has been successfully completed (below). We have requested a no-cost extension (submitted on July 2009) to complete the studies described in Aim 2 within the next year.

To address **Aim 1**, female Wnt-Tg mice at weaning [postnatal day (PND) 21] were randomly assigned to 1 of 2 semi-purified AIN93G-based isocaloric diets that differed only by protein source, namely CAS and SPI. Mice (n=22 for CAS; n=19 for SPI) were given *ad libitum* access to food and water. Mice were monitored for development of tumors by palpation starting at 10 weeks of age, and the age of initial appearance of tumors and initial tumor volume (measured by caliper) were recorded. Mice were necropsied two weeks after the initial appearance of a tumor to determine tumor growth rate. Tumor volume was recorded at tumor collection, and tumor pathology was scored by a board-certified pathologist (Dr. Leah Hennings) at the Histology Core Laboratory, Department of Pathology of the University of Arkansas for Medical Sciences.

Mice expressing *Wnt-1* under the control of the MMTV promoter develop extensive hyperplasias of the mammary gland (3, 4). **Figure 1** shows whole mounts of mammary glands harvested from virgin wildtype (WT) and Wnt-Tg mice of the same age (PND50) fed the SPI diet. While the WT mice exhibited normal mammary ductal morphogenesis, Tg mice displayed mammary gland hyperplasia with excessive ductal side-branching.

Tumor incidence in control CAS-fed Tg mice (11 of 22) was 50% while that for SPI-fed Tg mice (6 of 19) was 31.6%. Consistent with published reports (3), Tg mice fed CAS developed tumors within 5-7 months of age (6.54±0.48 months). SPI-fed Tg counterparts developed tumors earlier at 4.65±0.63 months (P=0.049, relative to CAS). Diet did not alter the rate of tumor growth, with CAS (81.63±9.16%) and SPI (83.33±2.95%) showing the same percentage increase in tumor volume 2 weeks after initial tumor detection. Finally, histopathological analyses of tumors from mice fed either CAS or SPI indicated tumors with comparable morphologic features (papillary adenocarcinoma, solid carcinoma with adenosquamous features). Collectively, these studies indicated that dietary intake of SPI relative to control diet CAS, is mammary tumor-protective in the Wnt-Tg mouse model of tumorigenesis. SPI diet reduced time of tumor onset, suggesting SPI effects on tumor progression, without affecting tumor size and tumor pathology. A summary of the evaluated tumor parameters as a function of dietary exposure to CAS or SPI is presented in **Table 1**.

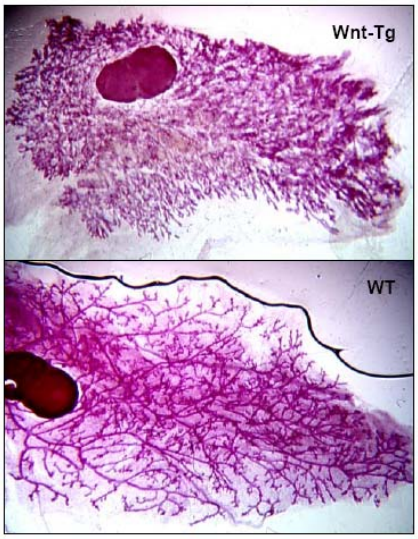

Figure 1. *Mammary ductal morphogenesis in Wnt-1-Tg mice compared to Wildtype mice. Whole mount staining was done on mammary glands harvested at PND50 mice fed the SPI diet beginning at weaning.*

To further evaluate how SPI may promote tumor progression coincident with reducing tumor initiation (i.e., lower tumor incidence relative to CAS diet), we evaluated dietary effects on the expression phenotype of a subset of genes in mammary tissues opposite and adjacent to sites of mammary tumors. PTEN and c-myc represent genes whose expression are altered during the development of tumors in Wnt-Tg mice, whereas Ly6a (Stem cell antigen, Sca-1), Keratin 6a/b (Krt6a/b), and Keratin 8 (Krt8) are considered markers of stem/progenitor cells (5). **Figure 2** shows that diet had no effect on the expression levels of all genes in mammary tissues in either location relative to the tumor. These results suggest that dietary effects on the expression of these genes may occur at an earlier stage during the development of mammary tumors. We will address this question in future studies by evaluating these genes' expression in hyperplastic mammary tissues collected at 3-4 months of age, prior to tumor development.

Table 1. Mammary Tumor Parameters as a Function of Diet

Diet	Mice/ Diet	Tumor Incidence[a]	Tumor Onset[b]	Tumor Growth rate[c]
CAS	22	50 %	6.54±0.48	81.63±9.16 %
SPI	19	31.6 %	4.65±0.63*	83.33±2.95 %

a Tumor Incidence = percentage of tumor-positive mice

b Tumor Onset = age at which tumor is initially detected, in months

c Tumor Growth Rate = percentage of tumor growth two weeks after tumor is initially detected

The above findings indicated that dietary SPI is tumor-protective in the mouse model of mammary tumorigenesis initiated by dysregulation of the Wnt signaling pathway. The mammary tumor protective effect of dietary SPI intake in Tg mice recapitulates that observed in the NMU- and DMBA-models of rat mammary carcinogenesis, where we found the same extent of protection (19-26% in rat models vs. 19% in Wnt-Tg mice) (6, 7). The slightly higher protection in the rat models may be due to the longer duration of exposure to dietary SPI (lifetime with rats vs. after weaning only in mice). Moreover, Tg mice express the initiating oncogene prior to dietary exposure as opposed to administration of tumor-initiating agents (DMBA, NMU) in rats after dietary SPI exposure was initiated. Nevertheless, Wnt-Tg mice constitute an excellent model for the study of stem cells/diet interaction because the tumors formed are homogenous and Wnt-initiated tumor stem cell markers (8) are well-characterized.

Aim 2 proposes to examine if the accumulation of mammary stem/progenitor cell population associated with increased Wnt signaling in Tg females is decreased with dietary intake of SPI relative to CAS. To address this, stem/progenitor cell population in premalignant (hyperplastic) mammary tissues from 10 week-old Tg mice fed CAS or SPI will be isolated using stem-progenitor cell-surface markers $Thy1^{+}CD24^{+}CD45^{-}$, following recently published studies (8). Mammary epithelial cells expressing these markers will be isolated by FACS and the percentage of isolated cells relative to total input cells will be quantified as a function of dietary CAS or SPI exposure from the resultant histograms.

We are in the process of assigning Tg mice to the two diets to initiate the analyses described above. Because it is necessary to pool mammary tissues from mice of the same dietary group for isolation of mammary epithelial cells and subsequent FACS analyses (4-5 mice/isolation), we are embarking on large-scale breeding of WT females mated to Tg males to generate the numbers of Tg females needed for the above studies. Given that the typical number of pups born for each breeding pair is 8-10 and that there is only 1 of 4 possibilities to obtain a female Tg mice, we anticipate that the above studies will take at least 6-8 months to complete.

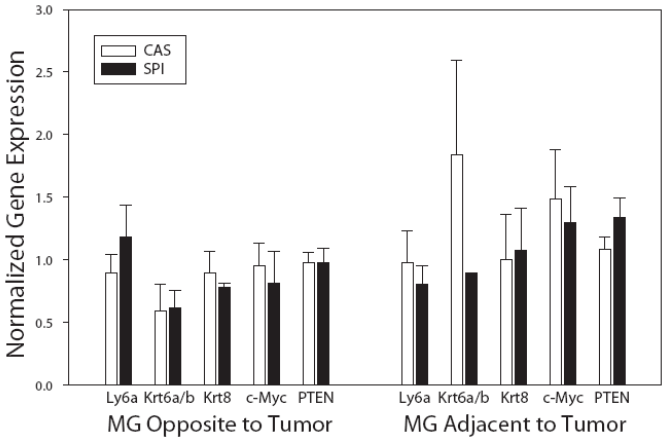

Figure 2. *Gene expression in mammary tissues opposite and adjacent to tumors of Wnt-1-transgenic mice fed either CAS or SPI beginning at weaning. n=6 mice/diet group.*

In preliminary studies to evaluate dietary factor effects on stem/progenitor cell population, we examined whether sera from young adult (PND50) rats lifetime exposed to dietary genistein beginning at gestation day 4 (through maternal diet) can influence the formation of mammospheres in cultures of human breast carcinoma cell line MCF-7, which have been demonstrated to contain cancer stem/progenitor cells (9). Genistein (GEN) is the major isoflavone found in soy foods and is considered to confer some of the dietary protective effects of SPI on mammary tumorigenesis (10, 11). We have previously shown that GEN at physiologically relevant levels (40 nM) can inhibit Wnt-signaling in the mouse mammary epithelial cell line HC11, by increasing the expression of the tumor suppressor E-cadherin and preventing Wnt-induced nuclear accumulation of β-catenin (12). The formation of non-adherent spherical clusters of cells, designated mammospheres, under anchorage-independent non-differentiating conditions is a hallmark of the tumor-forming potential of the multi-potent progenitor cell population. Our data indicated that sera from PND50 rats exposed to dietary GEN have decreased mammosphere formation efficiency than sera from control (CAS)-fed rats (**Fig. 3**). These findings are consistent with decreased expansion of the multi-potent progenitor cell population as a mechanism for mammary tumor protection by SPI, mediated by its major isoflavone GEN.

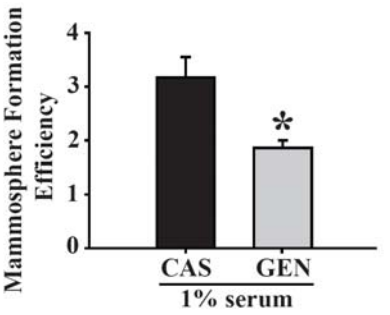

Figure 3. *Effect of serum from PND50 rats lifetime fed CAS or GEN on self-renewal potential of MCF-7 cells. GEN decreases mammosphere formation efficiency in MCF-7 cells relative to CAS serum. Values are mean ± SEM (n=3); *=P<0.05.*

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the Wnt-Tg mouse model of mammary carcinogenesis is a relevant model for investigating mammary tumor protection by diet.
- Demonstrated that SPI, the major protein component of soy foods (and soy milk formula) is mammary tumor protective at adulthood when consumed beginning at pre-puberty, indicating the significant influence of early (good) nutrition on mammary cancer risk.
- Provided data to support the concept that diet can modify systemic (serum) factors that can influence cancer stem/progenitor cell expansion.
- Established procedures in the laboratory (mammosphere assay, mammary epithelial cell isolation from tumors) for the further study of diet/stem cell interactions for breast cancer prevention and intervention.

REPORTABLE OUTCOME

- One scientific abstract on work supported by this award was presented in a poster platform at the Experimental Biology Meeting held in New Orleans in April 2009 (Simmen RC et al. 2009. Early Effects of Dietary Soy and Genistein in Rodent Models of Mammary Tumorigenesis, EB Meeting, New Orleans).
- One scientific abstract on work supported by this award has been accepted for poster presentation at the San Antonio Breast Cancer Symposium (San Antonio, TX) in December 2009 (Rahal O and Simmen RC. Induction of PTEN-p53 crosstalk in mammary epithelial cells: a novel mechanism of breast cancer prevention by the dietary factor genistein).
- Findings from this study will be used as preliminary/supporting data for a grant submission to NCI in Feb 2010.
- PhD student Omar Rahal and postdoctoral fellow John Mark Pabona, M.D., are currently supported in part by this award. Omar Rahal has submitted a grant application to the Department of Defense Pre-doctoral Fellowship Program on Breast Cancer, based in part on these funded studies.
- Dr. RCM Simmen will present an invited talk at the Arkansas Biosciences Institute Symposium set for September 25, 2009, and her talk will cover the Wnt-Tg mouse as a model of mammary carcinogenesis influenced by diet.

CONCLUSION

Our project tests the novel concept that cancer stem/progenitor cells in mammary tissues are targets of bioactive dietary factors, thus, dietary factors may confer protection from breast cancer by preventing the expansion of this unique cell population with tumorigenic potential. Our previous studies have demonstrated that bioactive components of soy foods (e.g., GEN) alter PTEN and E-cadherin/Wnt signaling pathways in mammary epithelial cells, consistent with their mammary tumor protective effects (12, 13). Given that PTEN and E-cadherin/Wnt signaling regulate stem/progenitor cell survival and renewal (1, 3, 8), our work provides a new paradigm on actions of dietary factors for breast cancer prevention. Studies to demonstrate a functional (inverse) connection between diets known to be protective against breast cancer in the human population and the abundance of cancer-initiating (stem) cells will lead to new early targets for treatments of breast and other types of cancers, to reduce tumor growth.

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APPENDICES

Appendix 1- Abstract to EB Meeting (2009)
Appendix 2- Abstract to SABC Symposium (2009)

SUPPORTING DATA- None

APPENDIX 1: Poster Presentation at the Annual Meeting of Experimental Biology 2009 (New Orleans)

Early Effects of Dietary Soy and Genistein in Rodent Models of Mammary Tumorigenesis

Rosalia CM Simmen[1,2], Ying Su[1,2], John Mark P Pabona[1], Omar Rahal[2,3], Christian Simmons[1], Leah Hennings[1,4].
[1]Physiology & Biophysics, [2]Interdisciplinary Biomedical Sciences, and [4]Pathology, University of Arkansas for Medical Sciences, and [2]Arkansas Children's Nutrition Center, Little Rock, AR 72202.

The risk of breast cancer is highly modifiable by diet. Breast cancer may have its origins during early mammary development, thus, the increasing popularity of soy food consumption among pregnant and breast-feeding women and early exposure to soy protein and bioactive components through soy infant formula could have significant implications on adult incidence of this disease. Since soy protein isolate (SPI) and genistein (GEN) diets decreased chemically-induced tumor incidence in adult female rats, dietary effects on genetic pathways underlying mammary tumorigenesis were evaluated. In rat mammary epithelial cells, SPI and GEN, relative to casein diet increased tumor suppressor PTEN and E-cadherin expression; these effects were recapitulated *in vitro* by GEN. Dietary SPI also decreased lipogenic gene expression in rat mammary stromal adipocytes *in vivo*, which was mimicked by GEN in 3T3-L1 adipocytes *in vitro*. Since Wnt signaling perturbation alters the epithelial hierarchy, MMTV-Wnt1 mice were investigated for dietary SPI and GEN effects on mammary progenitor cell population during disease development. Female mice at weaning were assigned to CAS, SPI- or GEN-based diets and mammary tumor incidence was monitored. Diet-mediated changes in mammary transcriptional programs and in epithelial subpopulations may underlie protection from developing mammary lesions. USDA-CRIS-6251-5100002-06S; DOD-BCRP.

APPENDIX 2: Accepted for Poster Presentation at the San Antonio Breast Cancer Symposium in December 2009 (San Antonio, TX)

Induction of PTEN-p53 crosstalk in mammary epithelial cells: a novel mechanism of breast cancer prevention by the dietary factor genistein

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Consumption of soy foods either at an early age or for lifetime has been associated with reduced risk for developing breast cancer in humans and in animal models. However, this association continues to be controversial and the precise mechanisms for protection remain elusive. Among the soy products, the isoflavone genistein (GEN) has been widely suggested to confer mammary tumor protection. Previously we demonstrated the increased expression of tumor suppressors PTEN and p53 in mammary epithelial cells (MECs) isolated from young adult female rats fed dietary soy protein isolate (SPI) or casein (CAS) supplemented with GEN, when compared to MECs from rats fed the control (CAS) diet. Since NMU-administered rats fed SPI had reduced tumor incidence and increased tumor latency than those fed CAS, PTEN and p53 likely mediate the observed tumor resistance with SPI *in vivo*. We hypothesized that GEN induction of PTEN and p53 in MECs results in the formation of a PTEN/p53 functional complex to negatively regulate breast cancer development. Here, we used the human non-tumorigenic, ER-negative mammary epithelial cell line, MCF-10A, as an *in vitro* system to mechanistically dissect ER-independent actions of GEN involving PTEN and p53. GEN (40 nM, 2μM) augmented PTEN and p53 expression in treated relative to control cells. GEN also induced nuclear co-localization and physical association of PTEN and p53. To test a functional consequence of GEN-induced PTEN/p53 cross-talk on mammary epithelial phenotype, we analyzed GEN effects on cell cycle progression and acini formation in 3D cultures. Our results showed attenuated cell proliferation and lower cyclin D1 and pleiotrophin transcript levels in GEN-treated cells, which were abrogated by small interfering RNA to PTEN, indicating PTEN-dependence. Using FACS analysis, we showed that GEN induced cell cycle arrest at G_0-G_1 phase. Treatment with GEN promoted early acini formation of MECs grown in Matrigel, which temporally coincided with PTEN-dependent suppression of p21 and p27 transcript levels. Further analyses of GEN effects on MECs demonstrated induction by GEN of PTEN promoter-luc reporter activity as measured by dual-luciferase assay. Interestingly, treatment with siRNA to either PTEN or p53 reduced basal and GEN-induced PTEN promoter activity. Given that p53 binds to the PTEN promoter, our results suggest a feed-forward cycle in which dietary factor (GEN) induction of nuclear PTEN leads to PTEN promotion of its own signaling. By maintaining a stable pool of nuclear p53 to boost its transcription, PTEN ensures its continuous expression in MECs to favor cell differentiation. These data elucidate a novel mechanism by which dietary factors with PTEN-inducing activity may attenuate breast cancer risk and development. Funding by USDA-CRIS 6251-5100002-06S and the Department of Defense Breast Cancer Program (0810548).